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14. ABSTRACT

Neurofibroma formation might require PI3K hyperactivation within the glial layer thatsurrounds motor neurons, and yet the signals regulating PI3K remain incompletely understood.We hypothesized that activation of the metabotropic glutamate receptor DmGluRA might activatePI3K in glia. In task #1, we proposed to test if inhibition of DmGluRA-PI3K activity inmotor neurons is sufficient to activate PI3K in the analogue of the Schwann cell called theperipheral glia (as monitored by perineurial glial growth). We found that that inhibitingPI3K activity by introducing the DmGluRA112b null mutation, or by expressing the PTEN or Foxotransgenes in motor neurons did significantly increase perineurial glial growth, whichsupports the hypothesis. In task #2, we proposed to determine if DmGluRA activity inperipheral glia is required for PI3K activation. We found that inhibiting DmGluRA activitypartly, but not completely, suppressed genotypes that increase perineurial glial growth,suggesting that DmGluRA as well as another neurotransmitter receptor regulate PI3K activity.

15. SUBJECT TERMS

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INTRODUCTION

Several lines of evidence have suggested that neurofibroma formation occurs at least in part via hyperactivation of PI3K, which is a direct target of Ras activity (Dasgupta et al., 2005; Johanessen et al., 2005; Lavery et al., 2007). The involvement of PI3K in neurofibroma formation suggests that molecules that participate in the regulation of PI3K in peripheral nerves might be promising targets for therapeutic intervention. However, our understanding of the mechanisms by which PI3K activity in the nervous system is regulated is incomplete. Recently, my lab showed that in Drosophila larval motor neurons, PI3K is activated by the group II metabotropic glutamate receptor DmGluRA (Howlett et al., 2008), raising the possibility that antagonists of these group II receptors might prevent PI3K activation and thus act therapeutically in neurofibroma formation. In this exploratory-hypothesis development award, I proposed two tasks to extend these observations. First (task one), I proposed to determine if inhibition of the DmGluRA-PI3K pathway in motor neurons would be sufficient to increase growth of the outer, perineurial glial layer (analogous to the mammalian perineurium). We found that this inhibition does indeed increase perineurial glial size, thus supporting this hypothesis. Second (task two), I proposed to determine if DmGluRA activity in peripheral glia (analogue of the Schwann cell) is required for increased perineurial glial growth. We found that DmGluRA knockdown specifically in the peripheral glia, mediated by RNAi, significantly, but incompletely, suppressed the increased perineurial glial thickness observed in one particular genotype: the *ine push* double mutant (Yager et al., 2001). I conclude that activity of DmGluRA in the peripheral glia is required in part for increased perineurial glia, but increased perineurial glial growth is capable of occurring even when DmGluRA is knocked down. These results suggest that glutamate-mediated DmGluRA activation in peripheral glia participates in PI3K activation, but that another pathway (possibly another neurotransmitter-mediated pathway) also participates.

BODY

Task one: Does the increased motor neuron excitability conferred by inhibiting the mGluRA-PI3K pathway promote perineurial glial growth?

In this aim, I proposed to inhibit DmGluRA-PI3K activity in motor neurons and, using electron microscopy, monitor the resulting effects on perineurial glial thickness. I proposed to perform these analyses both in a wildtype background, as well as in a background in which the *inebriated*-encoded neurotransmitter transporter was eliminated by chromosomal mutation. The use of the *ine* mutation was previously shown to sensitize the peripheral nerve to trophic effects of other mutations and to reveal effects on perineurial glial growth that were otherwise difficult to demonstrate. We used the motor-neuron specific *D42 Gal4* driver to express transgenes specifically in motor neurons.

We found that combining the ine^{-} mutation with genotypes that disrupt DmGluRA-PI3K activity in motor neurons does indeed significantly increase perineurial glial thickness (Figure 1A and Figure 1C). Changes in activities of three genes (*DmGluRA*, *PI3K* and *Foxo*) were previously shown to affect neuronal excitability (Howlett et al., 2008) and these are the three genes we altered to determine if their manipulations induced *ine*-mutant-dependent increased perineurial

glial growth. We found that *ine* mutants carrying the *DmGluRA* null mutation *DmGluRA*^{112b} exhibited perineurial glial thickness of 1.93 +/- 0.11 μm, which was significantly greater than control (*ine; D42>+*, which had a mean perineurial glial thickness of 1.47 +/- 0.14 μm, p=0.00005). Similarly, *ine; D42>PTEN* and *ine; D42>Foxo* significantly increased perineurial glial thickness (to 2.40 +/- 0.18 μm and 2.27 +/- 0.16 μm, p=0.000003 and p=0.00016, respectively).

In addition, we tested effects of a transgene that inhibits PI3K signalling more weakly than PTEN overexpression: the transgene *PI3K*^{DN}. We found that as anticipated, *ine; D42>PI3K*^{DN} larvae exhibited increased perineurial glial thickness, but to a lesser extent than the other genotypes tested (perineurial glial thickness was increased to 1.68 +/- 0.15 μm, p=0.048, Figure 1A). Taken together, these results confirm that blocking the DmGluRA-PI3K

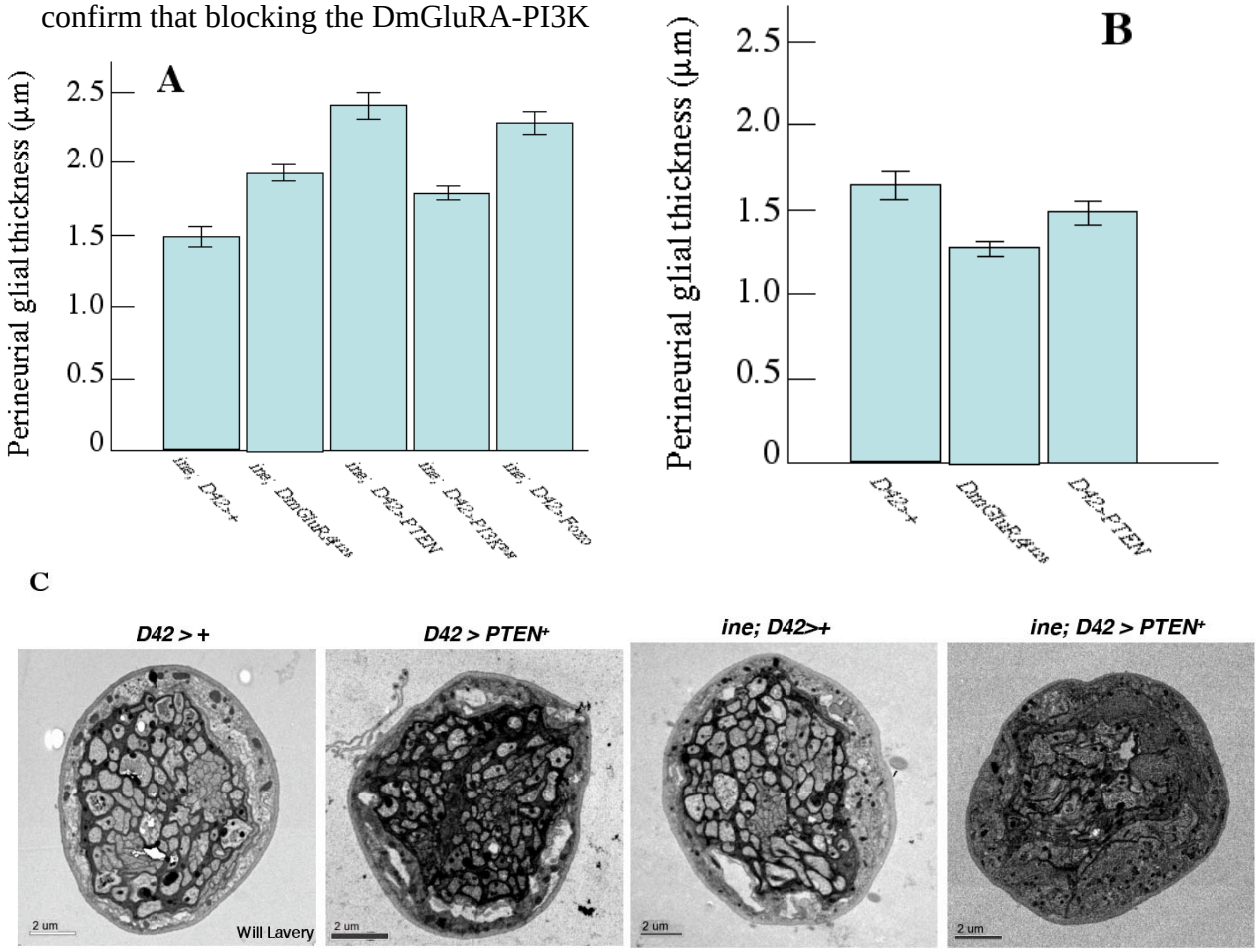


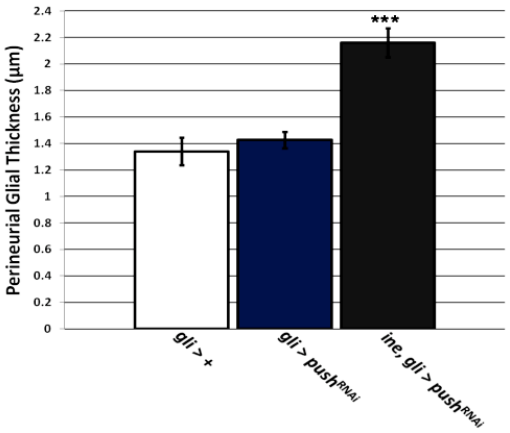
Figure 1: Increased neuronal excitability increases perineurial glial thickness in an *ine* mutant, but not *ine*$^{+}$, background. For panels A) and B), mean perineurial glial thickness (μm, +/- SEM) is shown along the Y axis for the genotypes indicated along the X axis. A) The following pairwise combinations had statistically significant differences (Student's unpaired t-test): For *ine; DmGluRA*^{112b} (lane #2, n=30), vs. *ine; D42>+* (lane #1, n=31), p = 0.00005; for *ine; D42>PTEN* (lane #3, n=26), vs. *ine; D42>*, p < 0.0001; for *ine; D42>PI3K*^{DN} (lane #4, n=21), vs. *ine; D42>+*, p=0.025; for *ine; D42>Foxo* (lane #5, n=31), vs. *ine; D42>+*, p=0.00015. B). For *D42>+*, n=48; for *DmGluRA*^{112b}, n=31; for *D42>PTEN*, n=26. Lower panels: Transmission electron micrographs from representative larval

peripheral nerves of the indicated genotypes. Increased perineurial glial thickness is observed only in *ine; D42>PTEN*, not *ine*$^{+}$*; D42>PTEN*. Scale bars as indicated.

pathway in motor neurons increases perineurial glial thickness in an *ine* mutant background.

To determine if this increased thickness was dependent on the *ine* mutation, we measured perineurial glial thickness in some of the genotypes listed above, except in an *ine*$^{+}$ background (Figure 1B). We found that as hypothesized, neither *DmGluRA*^{112b} nor *D42>PTEN* increased perineurial glial thickness in an *ine*$^{+}$ background (1.41 +/- 0.08 μm and 1.29 +/- 0.09 μm versus 1.67 +/- 0.11 for wildtype). We decided not to pursue studies on *D42>PI3K*^{DN} because the perineurial glial thickness in *ine; D42>PI3K*^{DN} was not substantially different from wildtype. Measurements on *D42>Foxo* were delayed and will be completed if a no-cost extension I applied for is approved. I conclude that the increased perineurial glial thickness observed when the DmGluRA-PI3K pathway is blocked requires the *ine* mutation.

Although the increased perineurial glial thicknesses shown in the genotypes in Figure 1 are significant, the magnitude of these increases are still less than the increases observed in other genotypes, such as in larvae doubly mutant for *ine* and a second gene called *push*, which encodes an E3 ubiquitin ligase homologous to mammalian UBR4 (Yager et al., 2001), and in larvae expressing the activated *PI3K-CAAX* in peripheral glia (Lavery et al., 2007): in both of these genotypes, perineurial glial thickness is 3.1 μm, rather than the 2.1 (or so) μm thicknesses observed in the genotypes described above. This observation led us to wonder if other factors, independent of neuronal excitability, might also contribute to perineurial glial growth. Because the *push* null mutation appears to confer a strong phenotype, and because the peripheral glia appears to be an important tissue for perineurial glial growth, we decided to test the possibility that *push* activity in the peripheral glia might contribute to perineurial glial growth. To test this possibility, we used a *push-RNAi* transgene and the peripheral glial-specific *Gal4* driver *gli-Gal4* to knockdown Push levels in the peripheral glia. Then we evaluated perineurial glial thickness in this genotype in both an *ine*$^{+}$ and *ine* mutant background. We found an increased perineurial glial thickness in *ine gli push-RNAi*, but not *ine*$^{+}$ *gli push RNAi*. Thus, loss of *push* in the peripheral glia increases perineurial glial thickness in an *ine* mutant background. We conclude that perineurial glial thickness is regulated both by motor neuron activity and by a push-regulated process in the peripheral glia.



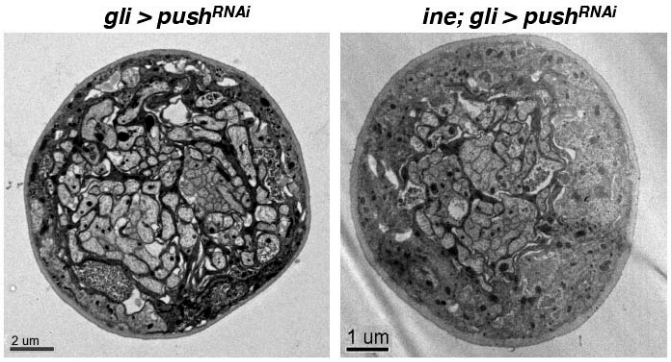


Figure 2: *push* knockdown in the peripheral glia confers *ine*-mutant-dependent increased perineurial glial thickness. Left panel: mean perineurial glial thickness (μm, +/- SEM) is shown along the Y axis for the genotypes indicated along the X axis. The following pairwise combinations had statistically significant differences (Student's unpaired t-test): For *ine; gli>push*^{RNAi} (lane #3, n=20), vs. *gli>push*^{RNAi} (lane #2, n=47), p <0.00001; For *ine; gli>push*^{RNAi} (lane #3, n=20), vs. *gli>+* (lane #1, n=25), p <0.00001; Right panels: Transmission electron micrographs from representative larval peripheral nerves of the indicated genotypes. Increased perineurial glial thickness is observed only in *ine; gli>push*^{RNAi}, but not *ine*$^{+}$*; gli>push*^{RNAi} . Scale bars as indicated.

Task two: Is DmGluRA activity in peripheral glia required for the ability of motor neuron activity to promote perineurial glial growth? As described above, in previous observations from my lab we found that *ine push* double mutant larvae exhibit greatly increased perineurial glial thickness. I hypothesize that the increased perineurial glial thickness in *ine push* double mutants occurs because the *ine push* genotype increases glutamate release from motor nerve terminals, thus hyperactivating PI3K in the peripheral glia via DmGluRA. If so, then blocking DmGluRA specifically in peripheral glia is predicted to block this PI3K hyperactivation and thus decrease perineurial glial thickness in *ine push*.

This hypothesis is not supported by the observation (Figures 1A and 1C) that increased perineurial glial thickness is observed in *ine; DmGluRA*^{112b}; that is, even in the absence of DmGluRA. Thus, DmGluRA activity is not absolutely required for increased perineurial glial growth in the presence of increased neuronal excitability and the *ine* mutation. However, I note that perineurial glial thickness in *ine; DmGluRA*^{112b} is decreased compared to *ine; D42>Foxo* and *ine; D42>PTEN* (Figure 1) even though neuronal excitability is greater in *DmGluRA*^{112b} than in *D42>Foxo* or *D42>PTEN* (Howlett et al., 2008). These observations raise the possibility that although *DmGluRA*^{112b} fails to eliminate the effects of neuronal excitability on perineurial glial growth, *DmGluRA*^{112b} is capable of attenuating these effects.

To address this hypothesis, we constructed two stocks. We first recombined the peripheral glial Gal4 driver *gli-Gal4* onto the *ine push* second chromosome to obtain a

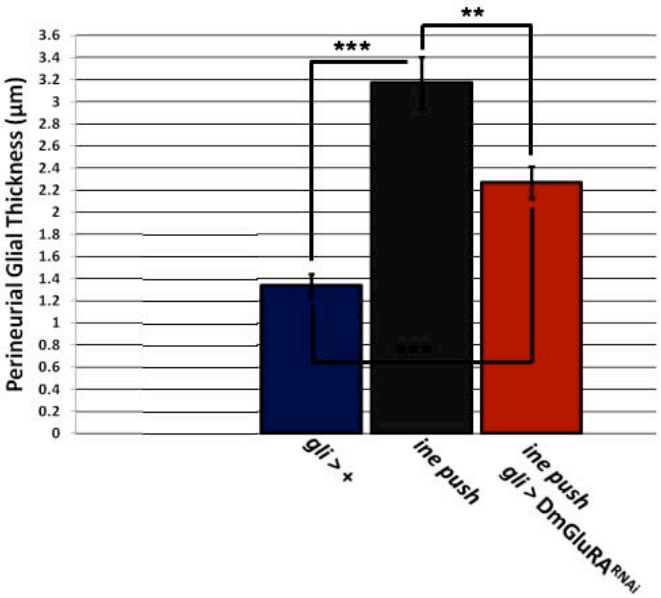


Figure 3: RNAi-mediated knockdown of *DmGluRA* in peripheral glia partially suppresses the increased perineurial glial thickness observed in *ine push* double mutants. Mean perineurial glial thickness (μm, +/- SEM) is shown along the Y axis for the genotypes indicated along the X axis. The following pairwise combinations had statistically significant differences (Student's unpaired t-test): For *ine push; gli>DmGluRA*^{RNAi} (lane #3, n=33), vs. *gli>+* (lane #1, n=25), p <0.00001; For *ine push; gli>DmGluRA*^{RNAi} (lane #3, n=25), vs. *ine push* (lane #2, n=16), p =0.0013.

chromosome carrying *ine push gli-Gal4*. In the second stock, we combined a second chromosome carrying *ine push* and a third chromosome carrying *UAS-DmGluRA-RNAi*. Because *push* mutations confer sterility, the second chromosomes of both stocks were balanced with the *CyO* balancer marked with GFP (to enable us to distinguish homozygous from balanced larvae). We crossed adults from the two stocks and assayed perineurial glial thickness in the larval progeny. We found that as predicted above, DmGluRA knockdown in the peripheral glia significantly, but incompletely, decreased the ability of the *ine push* double mutant to increase perineurial glial (Figure 3). These results suggest that DmGluRA within the peripheral glia is important for the glial response to increased neuronal excitability, but that another pathway (perhaps another neurotransmitter-mediated pathway) also participates.

KEY RESEARCH ACCOMPLISHMENTS

1) We have found that blocking DmGluRA-PI3K activity in motor neurons in an *ine* mutant background, but not an *ine*$^{+}$ background, increases perineurial glial thickness (Figure 1). These results support the first hypothesis of this proposal.

2) However, this increased thickness is less extreme than the increased thickness observed in the *ine push* double mutant. This observation raises the possibility that regulators in addition to the neuronal DmGluRA-PI3K pathway play roles in the control of perineurial glial thickness. Consistent with this possibility is the observation that blocking *push* in peripheral glia also increases perineurial glial thickness in an *ine* mutant-dependent manner (Figure 2). Taken together, these results suggest that perineurial glial thickness is regulated in part by neuronal activity and in part by a Push-regulated process within the peripheral glia.

3) Additional data reported has supported the second hypothesis in part: inhibition of *DmGluRA* within the peripheral glia suppresses in part the increased perineurial glial thickness of the *ine push* double mutant (Figure 3). This result indicates that DmGluRA within the peripheral glia is required in part for the ability of increased neuronal excitability to increase perineurial glial growth, and that another pathway (possibly another neurotransmitter-mediated pathway) distinct from DmGluRA also participates in the regulation of perineurial glial growth by neuronal activity.

REPORTABLE OUTCOMES

During 2010, Curtis Lin applied for and was invited to participate in two programs for senior graduate students contemplating postdoctoral work. The first program was at the NIH, and the second program was at University of Texas Southwestern Medical Center. Curtis has also applied for a program at the NIH entitled "Early independent scientists in the NIH intramural research program". He will be informed about his application status very soon.

CONCLUSIONS

Although generally not life-threatening, dermal neurofibromas can be painful and disfiguring and are thus important targets for therapy. Ras hyperactivation in peripheral nerve cell types as a consequence of loss of Nf1 is presumably causal for neurofibroma formation, but because very little is known about how Ras interacts with other genes to control peripheral nerve growth, there are no obvious targets for pharmacological intervention (except for Ras itself). In this project, and others from the lab that have been supported by the NFRP, we are trying to understand how growth within peripheral nerves is normally regulated at the molecular and cellular level; we hope that elucidating these growth controlling pathways will enable us to place Nf1 and Ras within identified pathways for which pharmacological targets can be chosen.

From funds provided for this project, we have demonstrated that motor neuron activity increases growth of the perineurial glial layer. Thus, pharmacological interventions that decrease neuronal activity might be therapeutic for neurofibroma formation. We also found that neuronal activity increases perineurial glial growth in part via the metabotropic glutamate receptor DmGluRA, and in part via a distinct pathway. Therefore targeting the metabotropic glutamate receptor might be therapeutic for neurofibroma formation, as originally hypothesized, and a second pathway, which remains to be identified, might also be a suitable target for pharmacological intervention.

PERSONNEL SUPPORTED

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